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The retinoblastoma protein (Rb) as an anti-apoptotic factor: expression of Rb is required for the anti-apoptotic function of BAG-1 protein in colorectal tumour cells

TJ Collard[1], BC Urban[1], HA Patsos[1], A Hague[2], PA Townsend[3], C Paraskeva[1] and AC Williams*,[1]

Although the retinoblastoma-susceptibility gene *RB1* is inactivated in a wide range of human tumours, in colorectal cancer, the retinoblastoma protein (Rb) function is often preserved and the *RB* locus even amplified. Importantly, we have previously shown that Rb interacts with the anti-apoptotic Bcl-2 associated athanogene 1 (BAG-1) protein, which is highly expressed in colorectal carcinogenesis. Here we show for the first time that Rb expression is critical for BAG-1 anti-apoptotic activity in colorectal tumour cells. We demonstrate that Rb expression not only increases the nuclear localisation of the anti-apoptotic BAG-1 protein, but that expression of Rb is required for inhibition of apoptosis by BAG-1 both in a γ-irradiated Saos-2 osteosarcoma cell line and colorectal adenoma and carcinoma cell lines. Further, consistent with the fact that nuclear BAG-1 has previously been shown to promote cell survival through increasing nuclear factor (NF)-κB activity, we demonstrate that the ability of BAG-1 to promote NF-κB activity is significantly inhibited by repression of Rb expression. Taken together, data presented suggest a novel function for Rb, promoting cell survival through regulating the function of BAG-1. As BAG-1 is highly expressed in the majority of colorectal tumours, targeting the Rb–BAG-1 complex to promote apoptosis has exciting potential for future therapeutic development.

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Evasion of apoptosis is a hallmark of cancer and contributes both to the development of the tumour and the refractory nature of tumours to treatment.[1] Understanding the molecular basis of resistance to apoptosis in colorectal cancer cells is essential if we are to identify novel targets for the prevention and treatment of colorectal cancer.

Retinoblastoma protein (Rb) is a well-established tumour-suppressor protein, regulating the transition of the restriction point in the cell cycle. Rb activity is also thought to be of importance in cell-fate determination; Rb is known to interact with lineage-specific transcription factors, including PU.1 and Id2 (erythroid development), Runx2 (bone development), MyoD (muscle) and Pdx1 (pancreatic development).[2,3] Further, loss of Rb function has recently shown to be sufficient to induce uncontrolled proliferation in stem cell populations.[4–6] However, apparently contradicting its role as a classical tumour-suppressor protein, colorectal cancer cells retain expression of the retinoblastoma tumour-suppressor protein (Rb); they express high levels of Rb compared with adjacent normal tissue, and loss or mutations of the *RB* gene are rare.[7,8] Furthermore, a study by Ali *et al.*[9] first revealed that colorectal tumours express entirely normal transcripts of retinoblastoma gene.[9] It is now recognised that Rb function is nearly always preserved and the RB locus sometimes even amplified in colorectal cancer.[10,11]

Much of the work on the function of Rb in colorectal carcinogenesis has centred on its ability to inhibit the E2F-dependent transcription. Of note, a recent study attributed the retention of Rb function in colorectal cancer cells to its ability to repress E2F-1-dependent inactivation of β-catenin signalling.[10,12] However, it is also well established that expression of Rb can promote cell survival through repression of E2F-driven apoptosis.[13] In fact, Rb was first recognised as anti-apoptotic in the *RB1*-knockout mice, which were found to be nonviable due to extensive apoptosis in neuronal and haematopoietic cells.[14–16] Further, Rb-deficient cells were reported to be more susceptible to apoptosis than cells with fully functional Rb,[17,18] and Rb expression inhibited cell death induced by DNA-damaging agents.[19,20] Although many studies have attributed the anti-apoptotic activity of Rb to repression of E2F-1 function, more recently Rb has also been reported to co-operate with E2F to activate pro-apoptotic genes in response to genotoxic stress.[21] Current evidence therefore suggests that the Rb status could be critical in influencing the sensitivity

[1]Cancer Research UK Colorectal Tumour Biology Research Group, School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK; [2]School of Oral and Dental Sciences, University of Bristol, Bristol, UK and [3]Cancer Sciences Division, University of Southampton, School of Medicine, Southampton General Hospital, Southampton, UK
*Corresponding author: AC Williams, Cancer Research UK Colorectal Tumour Biology Research Group, School of Cellular and Molecular Medicine, University of Bristol, Bristol BS8 1TD, UK. Tel: +44 117 3312070; Fax: +44 117 3312091; E-mail: Ann.C.Williams@bris.ac.uk
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Abbreviations: BAG-1, Bcl-2 associated athanogene 1; Dox, doxycycline; NF-κB, nuclear factor-κB; Rb, retinoblastoma protein; TNFα, tumour necrosis factor α
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of colorectal tumour cells to apoptosis. In this context, it is of interest that Rb has also been shown to interact with other proteins involved in regulating apoptosis, including the anti-apoptotic Bcl-2 associated athanogene 1 (BAG-1) protein, highly expressed in colorectal tumour cells.[22,23]

BAG-1 was discovered as a novel regulator of apoptosis through its ability to bind to Bcl-2.[24] Studies have since shown BAG-1 to be a multifunctional protein involved in a number of key cellular processes including proliferation, differentiation, cell cycle, transcription and apoptosis.[25,26] Characterised by the BAG domain, BAG-1 is a member of a family of related proteins, of which there are at least six in humans.[27] The human *BAG-1* gene encodes three BAG-1 isoforms, BAG-1S (p36), BAG-1M (p46) and BAG-1L (p50), generated via alternate translation mechanisms from a single mRNA.[28,29] The different BAG-1 isoforms have distinct subcellular localisations within the cell. Early studies reported that the smaller of the isoforms BAG-1S is preferentially located in the cytoplasm, the BAG-1M isoform is detected in both the nuclear and cytoplasmic compartments, and the BAG-1L isoform is located in the nucleus.[28,30–32] The difference in the subcellular localisation of the BAG-1 isoforms is thought to be conferred at least in part by a nuclear localisation signal present in the N-terminus of the BAG-1L isoform, but absent in the BAG-1S and truncated in the BAG-1M isoform.[28,31–33] Of note, contrary to the published literature, the localisation of the smaller BAG-1 isoforms in colorectal tumour cells appears distinct from other tissues, with the BAG-1M isoform being predominantly nuclear and the BAG-1S isoform exhibiting nuclear as well as cytoplasmic localisation.[34] The cumulative result is a predominant nuclear localisation of endogenous BAG-1 protein in colorectal epithelial cells (important for the transcriptional function of the protein[26]); previously associated with poor prognosis in colorectal cancer.[35]

BAG-1 is an important pro-survival protein in tumorigenesis; it has been shown to be overexpressed in a number of cancers[29] and to inhibit apoptosis in a variety of different cell types.[25] Overexpressed in colorectal adenoma and carcinoma tissue,[23] nuclear BAG-1 has been reported to correlate with poor prognosis[35] and promote tumour cell survival.[34] Interestingly, BAG-1 function has been linked to promoting the activity of the nuclear factor (NF)-κB pro-survival signalling pathway,[23] and was found to act as a co-regulator of gene expression through interaction with the p50-p50 NF-κB complexes,[36] suggesting a potentially important role for the BAG-1 NF-κB complex in colorectal carcinogenesis. Furthermore, recently it has been suggested that BAG-1 expression is the critical determinant in preventing c-MYC-induced apoptosis,[37] further emphasising the potential importance of BAG-1 function in colorectal carcinogenesis.

As we have previously established that the anti-apoptotic BAG-1 protein interacts with Rb, the question remained whether expression of Rb was implicated in the anti-apoptotic function of BAG-1 in tumour cells. Here we show for the first time that Rb expression is actually required for BAG-1 to inhibit γ-radiation-induced cell death both in Saos-2 osteosarcoma and in colorectal cancer cells. Consistent with the fact that BAG-1 had previously been shown to promote cell survival through increasing the activity of the NF-κB family of transcription factors,[23] the ability of BAG-1L to increase NF-κB activity was significantly inhibited on repression of Rb expression using an siRNA approach. Taken together, the data presented suggest a novel function for the Rb, promoting cell survival through its ability to promote the function of the BAG-1 protein. As BAG-1 is highly expressed in late-stage colorectal adenomas and colorectal carcinomas, targeting the Rb/BAG-1 cellular interaction may provide a selective mechanism to suppress the pro-survival functions of Rb and BAG-1 and increase the sensitivity of the colorectal cancer cells to current therapeutic regimes.

Results

Rb increases nuclear localisation and function of BAG-1 in osteosarcoma-derived Saos-2 cells. As Rb is expressed in colorectal cancer cells, it was hypothesised that Rb expression may contribute to tumour cell survival by regulating the function of the anti-apoptotic BAG-1 protein. Initial experiments were carried out to establish whether Rb is implicated in subcellular localisation and hence function of the BAG-1 protein. For these experiments, we used a model system to compare BAG-1 localisation and function in the presence and absence of Rb expression. The Saos-2 osteosarcoma cells (which are Rb null) have been used to generate an inducible Rb expression system, designated Saos-2/Rb/DC/R5, which contain a Tet/on inducible *RB1*-expression vector[38] (kind gift from S Weintraub, Washington, USA). Induction of Rb expression in the Saos-2 cells lead to an increase in the nuclear localisation of the endogenous BAG-1 protein (Figure 1a). To investigate whether this change in subcellular localisation was due to an interaction with Rb, BAG-1-binding (Rb-ABC) and non-binding (Rb-C) Rb fragments (developed and characterised[22]) were stably expressed in the Saos-2 cells and the subcellular localisation of the endogenous BAG-1 protein determined. Nuclear localisation of the Rb fragments was confirmed by cell fractionation (Figure 1bii). Expression of the BAG-1-binding fragment increased the nuclear localisation of endogenous BAG-1, whereas the non-binding fragment failed to change the localisation of the BAG-1 protein (Figure 1bi). Taken together, these data show that expression of Rb increases the nuclear localisation of BAG-1 protein in Saos-2 cells.

To determine whether Rb expression can regulate the nuclear activity of BAG-1, we assayed the ability of the Rb protein to increase BAG-1-dependent NF-κB activation (implicated in the pro-survival function of BAG-1[22]). In these experiments, we investigated the effect of Rb expression on endogenous BAG-1 activity; we knocked down BAG-1 activity using siRNA in the Saos-2/Rb/DC/R5 cells ($\pm$24 h doxycycline (Dox)). These cells were transfected with the NF-κB reporter construct, stimulated with tumour necrosis factor α (TNFα) (10 ng/ml) and activation of NF-κB determined after 16 h (Figure 1ci). Interestingly, NF-κB reporter activity was only significantly increased when endogenous BAG-1 levels were expressed in the presence of Rb protein (Figure 1ci, lane 4). Therefore, these results suggest that the expression of Rb in the Saos-2 cells not only increased the nuclear localisation of BAG-1 but also significantly increased BAG-1 nuclear function as shown by activation of NF-κB.

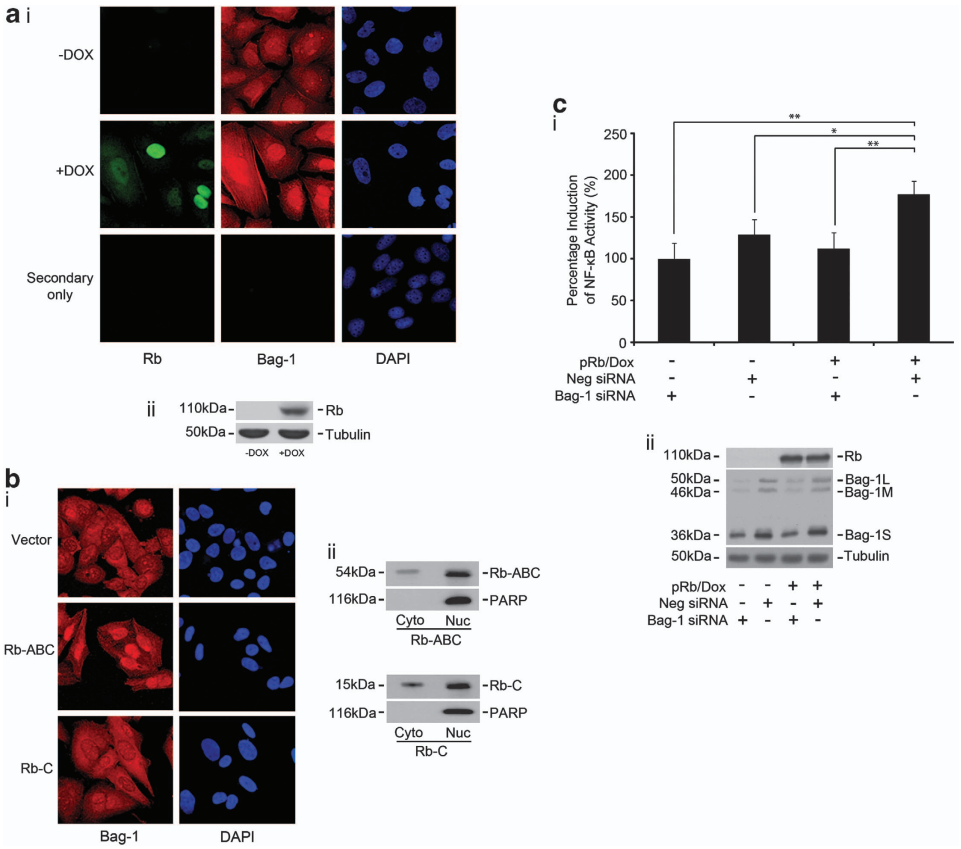

Figure 1 (**a**) Rb expression increases nuclear localisation of BAG-1 in Saos-2 cells. (ai) Confocal imaging showing Rb and BAG-1 expression in the Saos-2/Rb/DC/R5 ± 24 h treatment with 1 μg/ml Dox. DAPI was used to show nuclear staining. (aii) The induction of Rb expression on treatment with Dox was verified by western blot; α-tubulin was used as a loading control. (**b**) Expression of BAG-1 binding, but not the non-binding Rb fragment, increases the nuclear localisation of BAG-1 in Saos-2 cells. (bi) Confocal imaging of Saos-2 cells stably transfected with the pCDNA3.1 vector only, the Rb-ABC (BAG-1 binding) or the Rb-C (non-binding) fragments, suggesting that the effect of Rb binding regulates the subcellular localisation of the endogenous BAG-1 protein; DAPI was used to demonstrate nuclear staining. (bii) The nuclear localisation of both Rb fragments was confirmed by cellular fractionation. PARP was used as a nuclear protein control. (**c**) BAG-1 can only potentiate NF-κB activity in Saos-2 cells on expression of Rb protein. (ci) Induction of NF-κB activity, by luciferase reporter assay, stimulated with 10 ng/ml TNFα, for 16 h in Saos-2/Rb/DC/R5 cells. Results are expressed as a percentage increase over NF-κB activity in the TNFα-treated Rb null Saos-2/Rb/DC/R5 cells, in which endogenous BAG-1 expression has been suppressed using siRNA. The data represent the mean of three independent experiments ± S.D. and statistical differences determined by analysis of variance (ANOVA) with replication, $^{**}P<0.01$ and $^{*}P<0.05$. (cii) Western blot showing Rb expression in the Saos-2/Rb/DC/R5 cell line where the Rb protein is induced by the treatment of 1 μg/ml Dox (lanes 3 and 4), BAG-1 expression showing BAG-1 protein suppression by siRNA (lanes 1 and 3) and endogenous BAG-1 expression in the non-coding negative control (lanes 2 and 4). α-tubulin expression is shown as control for loading

Rb increases nuclear localisation and function of BAG-1 in colorectal cancer cells. As we were interested in Rb function in colorectal epithelial cells, we next determined whether expression of Rb was implicated in the predominant nuclear localisation of total BAG-1 protein in colorectal tumour cells. An RNAi approach was used to suppress Rb expression in the SW480 colorectal carcinoma cell line and confocal imaging to investigate the subcellular localisation of the BAG-1 protein (Figure 2a). It was found that when Rb expression was suppressed, there was a shift from the predominant nuclear localisation of the endogenous BAG-1 protein to a more even distribution of the BAG-1 protein throughout the cell (Figure 2a – results shown for one Rb siRNA sequence, parallel results were obtained for a second sequence, total BAG-1 expression levels did not change (Figure 2aii)). In addition, consistent with previous findings,[22] loss of Rb binding through expression of the HPV-E7 protein also decreased the overall nuclear localisation of endogenous BAG-1 protein in colorectal adenoma-derived cells (Figure 2b). Further, it is of interest to note that this *in vitro* finding models the *in vivo* localisation of the Rb and BAG-1 proteins in the normal colonic crypt. As shown in Figure 2c, expression of the Rb nuclear phosphoprotein at the bottom of the normal crypt (shown by the blue arrow) is coincident with predominant nuclear localisation of BAG-1 protein (shown by the red arrow). At the top of the crypt (towards the lumen), where Rb expression is downregulated,[39] the localisation of the BAG-1 protein is more cytoplamic (shown by the black arrow). Although correlative, the relative subcellular distribution of the proteins *in vivo* supports the *in vitro* findings that Rb increases the nuclear localisation of BAG-1.

To study whether Rb expression is also required for the enhancement of TNFα-induced NF-κB activity by BAG-1 in colorectal epithelial cells (as shown in Saos-2 cells,

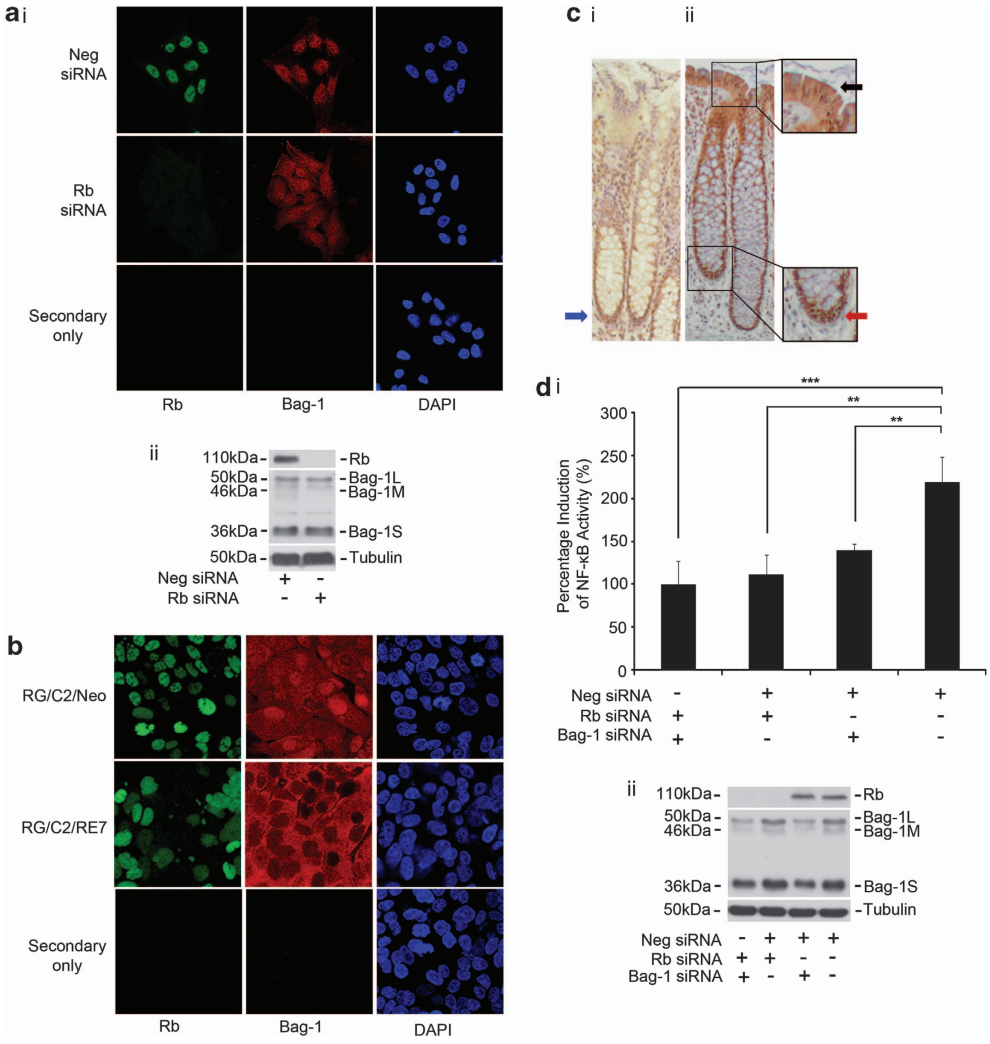

Figure 2 (**a**) Suppression of Rb expression in SW480 cells reduces the nuclear localisation of BAG-1 protein. (ai) Confocal imaging showing Rb and BAG-1 expression in SW480 cells where Rb expression has been suppressed using siRNA. DAPI was used to demonstrate nuclear staining. The secondary antibody only is shown to control for nonspecific staining. (aii) Western blot confirming reduction in Rb expression in the siRNA-treated cells. Of note, total BAG-1 levels are not regulated by suppression of Rb expression. α-tubulin is used as a loading control. (**b**) HPV-E7 expression decreases the overall nuclear localisation of endogenous BAG-1 protein in colorectal adenoma-derived cells. Confocal imaging showing Rb and BAG-1 expression in RG/C2 cells expressing either the HPV-E7 protein (RG/C2/RE7) or vector control (RG/C2/Neo). DAPI used to demonstrate nuclear staining. The secondary antibody only is shown to control for nonspecific staining. (**c**) Rb and BAG-1 protein expression in the normal colonic crypt. Tissue sections of normal colonic epithelium stained with the Rb antibody (ci), showing differential staining for Rb. The more intense staining is located at the bottom of the crypt (blue arrow) and with the TB-3 Bag-1 antibody (cii), showing that the nuclear BAG-1 staining is more intense at the bottom of the crypt (red arrow), and more cytoplasmic at the top of the crypt (black arrow). (**d**) Rb expression is required for the enhancement of TNFα-induced NF-κB activity by BAG-1 in SW480 cells. (di) Induction of NF-κB activity, by luciferase reporter assay, stimulated with 100 ng/ml TNFα, for 16 h in SW480 cells in which BAG-1 and/or Rb expression was suppressed by siRNA. Results are expressed as a percentage increase over NF-κB activity in the TNFα-treated SW480 cells, in which endogenous BAG-1 and Rb expression has been suppressed using siRNA. The data represent the mean of three independent experiments $\pm$ S.D., statistical differences determined by ANOVA with replication and Tukey's *post-hoc* test, $^{**}P<0.01$ and $^{***}P<0.001$. (dii) Western blot showing Rb and BAG-1 expression in the siRNA-transfected SW480 cells, where Rb expression is suppressed (lanes 1 and 2) and BAG-1 expression is suppressed (lanes 1 and 3) in both cases, compared with the non-coding negative control. α-tubulin expression is shown as control for loading

Figure 1c), we investigated the level of TNFα-induced NF-κB activity in SW480 cells, in which BAG-1 and Rb expression had been repressed (Figure 2d). This approach allowed us to determine the role of endogenous levels of Rb expression on the activity of BAG-1 in colorectal tumour cells. For these experiments, BAG-1 and/or Rb expression was suppressed by siRNA, cells were transfected with the NF-κB reporter construct and stimulated with 100 ng/ml TNFα (Figure 2d). Importantly, when BAG-1 and Rb proteins were expressed together in the cells, there was a significant increase in NF-κB activity (Figure 2di, lane 4). These findings suggest that both BAG-1 and Rb expression are required for increased TNFα-induced activation of NF-κB in colorectal epithelial cells as found in the Saos-2 cells.

Rb expression is required for the anti-apoptotic function of BAG-1L in γ-irradiated Saos-2 tumour cells. Previously, we have reported that BAG-1 inhibited γ-radiation-induced apoptosis in colorectal epithelial cells.[34] As Rb was found to increase the nuclear localisation and function of

BAG-1 protein (refer to Figure 1), we wanted to investigate whether expression of Rb in the Saos-2 cells was required for BAG-1-dependent inhibition of γ-radiation-induced apoptosis. To test this, BAG-1L was expressed in the Saos-2/Rb/DC/R5 ($\pm$ Dox) cells treated with 10 Gy radiation, and apoptosis was assessed (demonstrated by cleavage of caspase 3 and PARP, as described in detail previously,[40,41] Figure 3aiii). Interestingly, despite achieving high expression levels (Figure 3aii), the exclusively nuclear-localised BAG-1L isoform was only able to significantly inhibit radiation-induced apoptosis in the Dox-treated Saos-2/Rb/DC/R5 cells when Rb is expressed (Figure 3ai, lane 4), consistent with a requirement for Rb expression for the anti-apoptotic function of BAG-1L. These data highlight that nuclear localisation of even high levels of BAG-1 is insufficient to inhibit radiation-induced apoptosis in the absence of Rb protein expression, and emphasise that Rb expression is required for the inhibition of radiation-induced apoptosis by BAG-1 in Saos-2 cells.

Rb expression is required for the anti-apoptotic function of BAG-1L in colorectal tumour cells. As we are interested in the regulation of DNA damage-induced apoptosis in colorectal tumour cells, it was important to determine whether Rb expression was also required for the anti-apoptotic activity of BAG-1 in colorectal epithelial cells.[34] To determine the function of Rb, the apoptotic response of the irradiated SW480 cells was compared with those in which BAG-1 and Rb expression had been repressed (Figure 4a). Cells were transfected with BAG-1 and Rb siRNAs or equivalent non-coding negative control; the induction of apoptosis 72 h after 5 Gy γ-irradiation is shown (results shown for single sequences, parallel results were obtained for additional sequences, data not shown). Expression of endogenous levels of BAG-1 in the presence of Rb (Figure 4ai, lane 4) resulted in a significant reduction of apoptosis in the irradiated SW480 cells; further highlighting that BAG-1 and Rb function together to suppress γ-radiation-induced apoptosis in colorectal carcinoma-derived SW480 cells.

To confirm that the interaction between Rb and BAG-1 is required to protect the cells from radiation-induced apoptosis, we studied the radiation sensitivity of RG/C2 cells stably expressing HPV-E7, previously reported to interrupt the Rb/BAG-1 interaction. Of note, expression of the HPV-E7 protein did not lead to degradation of Rb protein in these cells (Figure 4bii), as previously described.[22] As HPV-E7 expression had previously been shown to block the BAG-1L–Rb complex formation, we were able to address the question whether nuclear BAG-1 requires interaction with the Rb protein to repress apoptosis in the cells. Cells were exposed to 5 Gy γ-radiation, and apoptosis was assessed 72 h after treatment (Figure 4b). Importantly BAG-1L overexpression was able to protect the cells against radiation-induced apoptosis in the control RG/C2/Neo cells (wild-type Rb function). In contrast, expression of the E7 protein blocked the anti-apoptotic activity of the BAG-1L protein (Figure 4bi), supporting the hypothesis that the interaction between Rb and

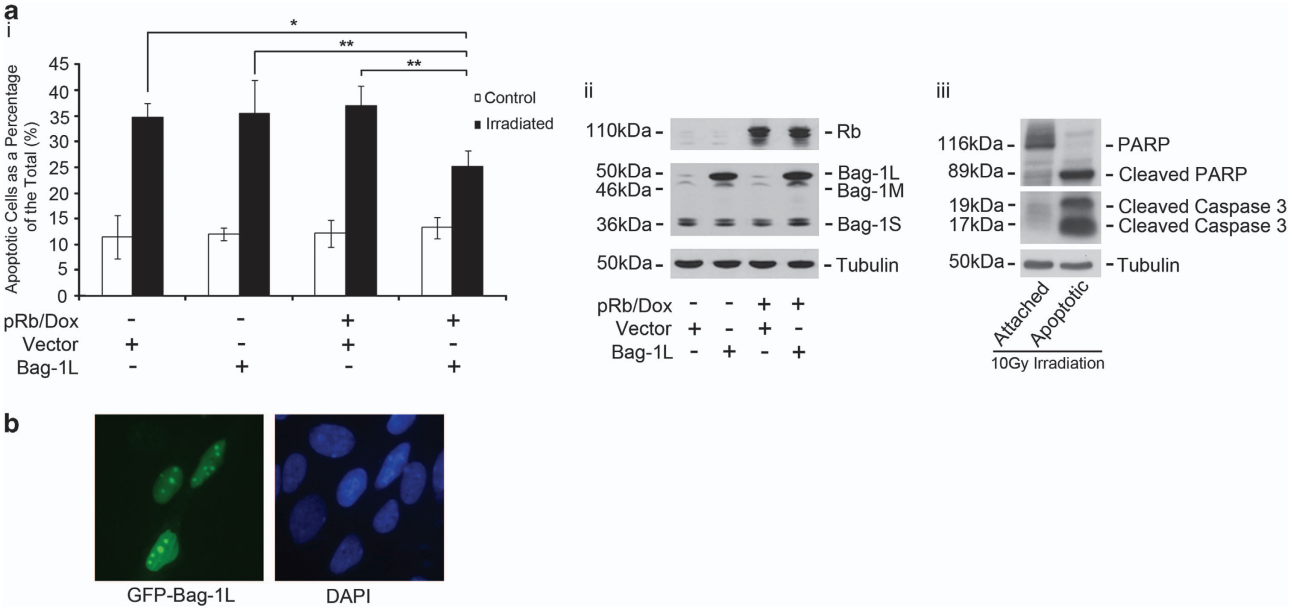

Figure 3 (**a**) Overexpression of the nuclear-localised BAG-1L isoform only inhibits radiation-induced apoptosis in Saos-2 cells on expression of Rb protein. (ai) Induction of apoptosis, in the Saos-2/Rb/DC/R5 cell line, 72 h after exposure to 10 Gy γ-irradiation. Cells were stably transfected with the empty Iresneo2 expression vector or Bag-1L, with or without expression of Rb protein after treatment with 1 μg/ml Dox. The results represent the means of three independent experiments $\pm$ S.D., statistical differences determined by ANOVA with replication and Tukey's *post-hoc* test, $^{**}P<0.01$ and $^{*}P<0.05$. (aii) Western blot showing Rb expression in the non-induced (lanes 1 and 2) or Dox-treated (1 μg/ml) Saos-2/Rb/DC/R5 (lanes 3 and 4), BAG-1 expression in the cells stably transfected with either the Iresneo2 vector control (lanes 1 and 3) or Bag-1L (lanes 2 and 4). α-tubulin expression is shown as a loading control. (aiii) Western blot showing cleaved PARP and caspase 3, confirming apoptosis in the irradiated cells. α-tubulin expression is shown as a loading control. (**b**) Expression of the GFP-tagged BAG-1L isoform demonstrating nuclear localisation of the protein. Saos-2 cells transiently transfected with the GFP-tagged Bag-1L, confirming nuclear localisation of the exogenous BAG-1L protein in the absence of Rb expression. Nuclear staining shown by DAPI

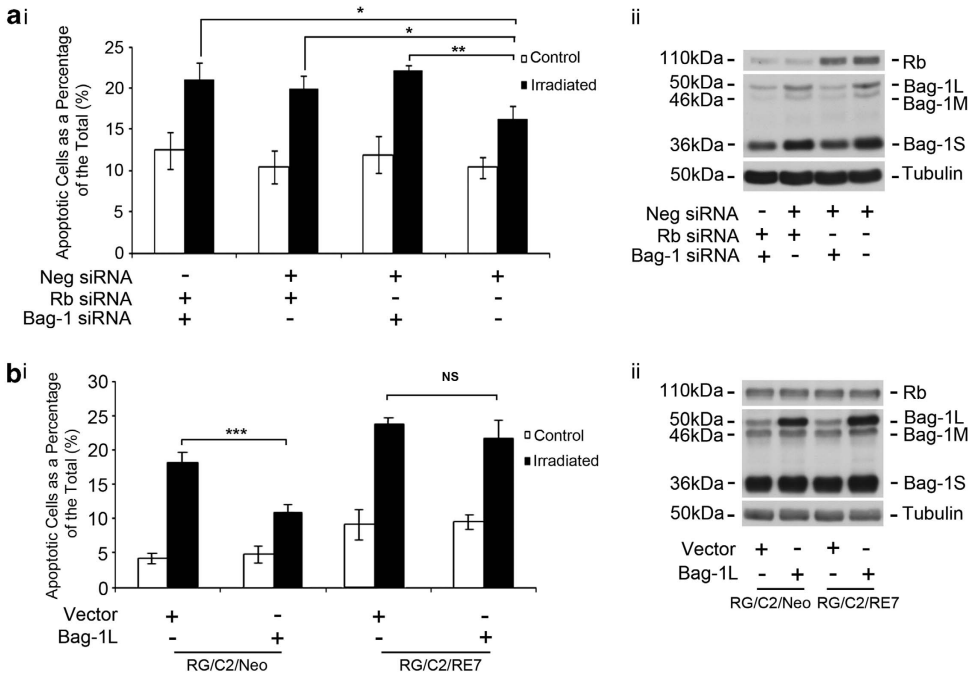

Figure 4 (**a**) Rb expression is required for the anti-apoptotic function of BAG-1 in irradiated SW480 cells. (ai) Induction of apoptosis in the SW480 cell line 72 h after exposure to 5 Gy γ-irradiation. Cells were transiently transfected with both Rb and Bag-1 siRNA 72 h before being exposed to irradiation. Apoptosis was compared in cells transfected with Rb, Bag-1 and/or the negative control siRNA. The results represent the means of three independent experiments $\pm$ S.D., statistical differences determined by ANOVA with replication and Tukey's *post-hoc* test, $^{**}P<0.01$ and $^{*}P<0.05$. (aii) Western blot showing Rb and BAG-1 expression in SW480 cells treated with Rb siRNA (lanes 1 and 2) and/or BAG-1 siRNA (lanes 1 and 3). Controls were transfected with the relevant non-coding negative siRNA. α-tubulin expression was used as a loading control. (**b**) The Rb–BAG-1 interaction is required for the anti-apoptotic function of Bag-1 in RG/C2 colorectal adenoma-derived cells. (bi) Induction of apoptosis in the RG/C2/E7 and RG/C2/Neo cell lines 72 h after exposure to 5 Gy γ-irradiation. Cells were stably transfected with Iresneo2 (vector) or expressing Bag-1L. The results represent the means of three independent experiments $\pm$ S.D., statistical differences determined by ANOVA with replication and Tukey's *post-hoc* test, $^{***}P<0.001$. (bii) Western blot showing Rb and BAG-1 expression in the RG/C2/RE7 cell line and control RG/C2/Neo. Cells stably transfected with either Bag-1L (lanes 2 and 4) or the Iresneo2 vector control (lanes 1 and 3). α-tubulin expression was used as a loading control. Of note, stable expression of the HPV-E7 protein did not decrease the level of Rb expression in these cells (as previously reported[22])

BAG-1 is required for the pro-survival role of BAG-1L in irradiated cells.

Discussion

Rb acts as a critical co-ordinator in cells, its multiple roles ensuring cellular and tissue homoeostasis.[11] Although a well-established tumour-suppressor protein, intriguingly Rb expression is not lost in colorectal carcinogenesis,[10,11] suggesting that Rb function is important for colorectal tumour development. In fact, Rb has been reported to act as a pro-survival factor in a number of different cell types. Hence retaining Rb expression may be beneficial for other cancers as well as colorectal cancer, at least until the tumour cells have acquired other mutations that block the cell-death pathways.[11] However, the mechanism through which Rb can support tumorigenesis remains to be fully elucidated. In this context, our previous finding that Rb interacts with the anti-apoptotic BAG-1 protein was of interest. As BAG-1 has been reported to be an important survival factor in a number of different cancers, including colorectal cancer,[23,29] we proposed that Rb expression could increase the anti-apoptotic function of BAG-1. Excitingly, data presented in this study show that Rb expression is actually required for the anti-apoptotic function of BAG-1, not only in colon tumour-derived cell lines but also in the Saos-2 osteosarcoma cell line. This finding not only highlights a novel role for Rb, promoting the anti-apoptotic function of the BAG-1 protein in colorectal tumour cells, but also implies that the Rb–BAG-1 complex may be important in a wider context, promoting the aberrant survival of specific tumour cell types where Rb is not acting as a classical tumour-suppressor gene.

Interestingly, in colorectal cancer cells, it has been reported that Rb promotes tumour development by preventing the inhibition of β-catenin transcription by E2F-1.[10,12] E2F-1 deregulation has been shown to suppress β-catenin activity in an adenomatous polyposis coli (APC)/glycogen synthase kinase-3 (GSK3)-independent manner, reducing the expression of key β-catenin targets, including c-MYC. It has been suggested that this interaction explains why colorectal tumours, which depend on β-catenin transcription for their abnormal proliferation, keep *RB1* intact.[10] In the current paper, we show that Rb function is also required for the anti-apoptotic function of BAG-1 and propose that in addition to regulating E2F-1 activity, Rb may also influence colorectal tumour cell fate through interaction with BAG-1. This finding is of further significance for colorectal carcinogenesis in light of a recent report that BAG-1 expression protects cells from c-MYC-induced apoptosis. It was reported that blocking BAG-1 was sufficient to convert cells from MYC-driven proliferation to MYC-induced

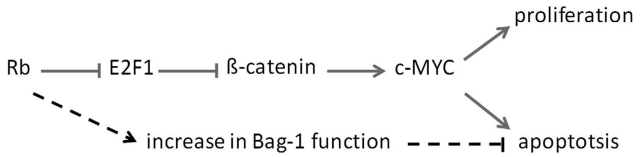

Figure 5 Schematic of pro-tumorigenic roles of Rb in colorectal cancer (solid line, from studies;[10,12,37] dotted line, regulation of function). Schematic representation of the proposed dual role for Rb in colorectal tumour cells: as well as inhibiting E2F-1 activity (blocking the inhibition of wnt signalling and hence increasing β-catenin activity), Rb is further required to promote the anti-apoptotic function of BAG-1 in the context of deregulated c-MYC activity in colorectal tumour cells

apoptosis.[37] Therefore, as c-MYC is deregulated early in colorectal carcinogenesis following β-catenin activation, we suggest that Rb expression could also be critical for the inhibition of MYC-induced apoptosis through promoting the pro-survival activity of BAG-1 in colorectal tumour cells. Therefore, we would propose that, as well as inhibiting E2F-1 activity (blocking the inhibition of wnt signalling and hence potentiating β-catenin signalling), Rb may further be required to promote the anti-apoptotic function of BAG-1 because of the ensuing deregulated c-MYC activity in colorectal tumour cells (Figure 5). Hence, it is possible that it is the ability of Rb to promote the anti-apoptotic function of BAG-1 that is a critical determinant for retaining Rb expression in tumours with deregulated c-MYC activity, promoting c-MYC-driven tumorigenesis and blocking MYC-induced apoptosis in the early stages of colorectal carcinogenesis.

Given its key anti-apoptotic role in a number of important tumours, there has been significant interest in inhibiting the pro-survival function of BAG-1 in cancer cells. Although studies investigating the targeting of BAG-1 function therapeutically are in their infancy, a short inhibitory peptide that blocks association with HSP70 binding, crucial for many of BAG-1's pro-survival functions, has been described.[42] Furthermore, the same group has identified a small molecule inhibitor, thioflavin S, which also possesses the capacity to bind and inhibit the C terminus of BAG-1. Of particular interest was the fact that both the short peptide and thioflavin S can inhibit cell growth in a number of high BAG-1-expressing breast cancer cell lines,[43] supporting the premise that inhibition of BAG-1 function has therapeutic potential in a number of tumour types. However, results from this study suggest a more specific way of targeting BAG-1 function in the cancer cells. As Rb expression was found to be required for the anti-apoptotic function of BAG-1, disrupting the Rb–BAG-1 interaction (which is independent of HSP70 binding[22]) would be expected to specifically inhibit the pro-survival function of BAG-1, but not many of the HSP70-dependent activities of BAG-1, potentially important in normal tissue homoeostasis. Hence, targeting the Rb–BAG-1 interaction has the potential to specifically inhibit the anti-apoptotic function of BAG-1 in tumour cells, and therefore represents a potentially important novel target for the treatment of colorectal cancer. Further, as we have demonstrated the Rb–BAG-1 co-operation in inhibiting apoptosis in the Saos-2 osteosarcoma cell line as well as in colorectal tumour cells, we can speculate that such an approach may have a wider application, increasing the sensitivity of a number of cancers to current therapeutic regimes.

Materials and Methods

Cell lines and culture conditions. The human colonic adenoma-derived S/RG/C2 cell line stably infected with the HPV-E7 oncoprotein (designated RG/C2/RE7 and corresponding vector control RG/C2/Neo) was maintained on conditioned medium with neomycin (G418) (Sigma, Poole, UK) at a concentration of 200 μg/ml (previously described[34]). Both the Rb null Saos-2 osteosarcoma cells (American Type Culture Collection, Manassas, VA, USA) and the strain Saos-2/Rb/DC/R5,[38] which contains a Tet/on inducible vector expressing Rb (kind gift from S Weintraub, WA, USA), are routinely cultured in DMEM (PAA, Yeovil, UK) supplemented with 100 U/ml penicillin (Invitrogen, Paisley, UK), 100 μg/ml streptomycin (Invitrogen), 2 mM glutamine (Sigma) and 15% fetal bovine serum (FBS) (PAA). Rb expression was induced in the Saos-2/Rb/DC/R5 cells by 24-h treatment with 1 μg/ml Dox (Sigma). The human colonic carcinoma-derived cell line SW480 was obtained from American Type Culture Collection and cultured in 10% FBS DMEM, as previously described.

Stable transfections. The parental Saos-2 cell line was transfected using Genejuice (Novagen, Merck, Nottingham, UK), according to the manufacturer's instructions with one of the three plasmids, either a region of Rb that binds BAG-1, designated Rb-ABC, the non-binding C-fragment, Rb-C or the vector-only control pCDNA3.1 (Invitrogen). The clones were selected using 400 μg/ml G418 (Sigma) and maintained on 15% FBS DMEM containing 200 μg/ml G418. Verification of their expression was confirmed by western blotting.

Both the retroviral-infected S/RG/C2 HPV-E7 expressing cells and the inducible Saos-2/Rb/DC/R5 cells were stably transfected with the nuclear-localised BAG-1L isoform (gift from G Packham, Southampton, UK) or the pIRESneo2 vector only (Clontech, Mountain View, CA, USA) using Lipofectamine 2000 (Invitrogen) or Genejuice for the Saos-2/Rb/DC/R5 cells as per the manufacturer's instructions. Pooled colonies were maintained on their respective growth medium containing 200 μg/ml G418. Verification of BAG-1L expression was established by western blotting.

Confocal imaging. Cells were seeded on 19-mm coverslips, in six-well plates, and grown for 3 days before being fixed with 4% paraformaldhyde (Sigma) and Triton-X (Sigma). To induce Rb expression in the inducible Saos-2/Rb/DC/R5, Dox treatment, 1 μg/ml, was applied 24 h before fixture. The cells were dual stained with the mouse Rb antibody (BD Pharmingen Europe, Erembodegem, Belgium) and rabbit BAG-1 antibody TB-3 (gift from Graham Packham, Southampton, UK), both used at a dilution of 1 : 100. Visualisation was achievable using Alexa 488 (green) anti-mouse (Invitrogen) at a dilution of 1 : 100 and Alexa 546 (red) anti-rabbit at 1 : 200.

siRNA transfection (SW480 and Saos-2/Rb/DC/R5). SW480 cells were seeded grown to 50% confluent under the standard conditions for 72 h, before being transfected using Lipofectamine 2000, as per the manufacturer's instructions. Suppression of Rb and/or BAG-1 protein was achieved by siRNA oligonucleotides (Applied Biosystems, Carlsbad, CA, USA) at a final dilution of 50 nM siRNA concentration, controlled with a negative sequence, with no homology to any sequence in the human genome. A total of 50 nM siRNA was used to achieve reliable suppression of BAG-1 protein as previously published.[23,36] The following sequences were used: Rb sequence 5′-GGUUCAACUACGCGUGUAAtt-3′ and BAG-1 sequence 5′-GGGAAAAUCUCUGAAGGAAtt-3′. The Saos-2/Rb/DC/R5 cells were transfected with the BAG-1 siRNA using the above method, and Rb expression was controlled by treatment with 1 μg/ml Dox.

SDS-PAGE – western blotting. Cell samples for western blotting were prepared by lysing cells for 15 min in lysis buffer (Cell Signaling, Danvers, MA, USA) containing Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Cell debris was removed by centrifugation at 18 500 r.p.m. for 10 min at 4 °C. A Bradford assay was used to establish the concentration of samples; 100 μg total protein was diluted in Laemali buffer and loaded per lane using the standard techniques. Proteins were resolved on a 10% gel and transferred on to Immobilon-P membrane PVDF (Millipore, MA, USA). Primary antibodies were incubated overnight; Rb (BD Pharmingen Europe) at 1 : 1000, BAG-1 and G3E2 (gift from G Packham, Southampton, UK) at 1 : 100 and α-tubulin (Sigma) as a loading control at a dilution of 1 : 10 000. All proteins were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Sigma), and bands were visualised by chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

Treatment with γ-irradiation. Cells were treated at 70% confluence, 24 h after Dox induction of Rb or 72 h after transient transfection of siRNA. Duplicate flasks of infected S/RG/C2, SW480 or inducible Saos-2 cells were exposed to either 5 or 10 Gy, from a ^{137}C source at a dose rate of 0.33 Gy/min, with 20 mM Hepes (Sigma) for buffering. The γ-radiation dose used was determined by the different radiation sensitivity of the cell lines; the dose was adjusted to achieve 25–30% apoptosis in untransfected cells: S/RG/C2 and SW480 cells were treated with 5 Gy and the Saos-2-derived cells with 10 Gy. Attached and floating cell counts were determined 72 h after irradiation.

Assessment of apoptosis. The level of apoptosis was determined as previously described. Cells were confirmed as apoptotic by changes in morphology as detected by acridine orange staining and cleavage of caspase 3 and PARP, as described in detail previously.[34,40]

NF-κB reporter assay (Saos-2/Rb/DC/R5 and SW480). Cells were transiently transfected with either the NF-κB reporter plasmid pNF-κB-TA-luc or the control plasmid pTA-luc (Clontech, BD Europe, Erembodegem, Belgium) as previously described.[23] In brief, the Saos-2/Rb/DC/R5 cells were grown to 50% confluency, under the standard conditions for 72 h, before being co-transfected with 2.5 μg per flask of either reporter constructs and a 50-nM final dilution of BAG-1 siRNA oligonucleotide or the negative control. After 24 h, the cells were treated with 1 μg/ml Dox, where necessary to induce Rb expression. The SW480 cells were treated with 50 nM Rb and/or BAG-1 siRNA or negative control oligonucleotides as described above, followed by transfection with the pNF-κB-TA-luc or control pTA-luc plasmid after 48 h. To stimulate NF-κB activity, cells were further treated for 16 h with 10 ng/ml TNFα (Saos-2/Rb/DC/R5) or 100 ng/ml TNFα (SW480).

GFP imaging. Saos-2 parental cells were seeded on 19-mm coverslips, in six-well plates, and grown for 3 days before being transiently transfected as per the manufacturer's instructions, using Genejuice to introduced pEGFP-Bag-1L (gift from G Packham)[34] in the cells. After a further 24 h under normal growth conditions, the cells were fixed with 4% paraformaldhyde and Triton-X, and observed under fluorescence.

Immunostaining of normal colonic crypts. Sections were prepared from archival material retrieved from files at the Department of Histopathology, Bristol Royal Infirmary, UK, with local Ethic Committee approval. Normal mucosa was obtained from resection margins at least 6 cm from the tumour mass. Sections were stained using Rb antibody (BD Pharmingen Europe) at a dilution of 1 : 1000 or BAG-1 antibody, TB-3 (gift from G Packham) used at a dilution of 1 : 1400.

Statistical analysis. Statistical analysis was carried out using SPSS statistical software for Windows (version 19; SPSS Inc., Chicago, IL, USA). Analysis of variance was used to determine differences among the means. The experiments were repeated three times and the results were presented as a mean of the three separate experiments. Pairwise comparisons were made using Tukey's *post-hoc* test for multiple comparisons.

Conflict of Interest

The authors declare no conflict of interest.

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